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Simple Colorimetric Trypanothione Reductase-Based Assay for High-Throughput Screening of Drugs against *Leishmania* Intracellular Amastigotes

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Critical to the search for new anti-leishmanial drugs is the availability of high-throughput screening (HTS) methods to test chemical compounds against the relevant stage for pathogenesis, the intracellular amastigotes. Recent progress in automated microscopy and genetic recombination has produced powerful tools for drug discovery. Nevertheless, a simple and efficient test for measuring drug activity against *Leishmania* clinical isolates is lacking. Here we describe a quantitative colorimetric assay in which the activity of a *Leishmania* native enzyme is used to assess parasite viability. Enzymatic reduction of disulfide trypanothione, monitored by a microtiter plate reader, was used to quantify the growth of *Leishmania* parasites. An excellent correlation was found between the optical density at 412 nm and the number of parasites inoculated. Pharmacological validation of the assay was performed against the conventional alamarBlue method for promastigotes and standard microscopy for intracellular amastigotes. The activity of a selected-compound panel, including several anti-leishmanial reference drugs, demonstrated high consistency between the newly developed assay and the reference method and corroborated previously published data. Quality assessment with standard measures confirmed the robustness and reproducibility of the assay, which performed in compliance with HTS requirements. This simple and rapid assay provides a reliable, accurate method for screening anti-leishmanial agents, with high throughput. The basic equipment and manipulation required to perform the assay make it easy to implement, simplifying the method for scoring inhibitor assays.

The global scope of leishmaniasis and the inadequacy of present treatments support the need for improved chemotherapies (1, 2). Undermined by severe toxicity and high failure rates, besides poor compliance and high costs, current anti-leishmanial treatment relies on a few drugs, of which toxic antimonials remain the mainstay 70 years after their introduction (3). Assessment of novel drugs or treatment regimens for leishmaniasis requires reliable methods for high-throughput screening (HTS) against the clinically relevant stage of the disease, the intracellular amastigotes (4, 5). For decades, anti-leishmanial drug discovery has rested upon the use of insect stage, free living promastigotes, for which simple and efficient *in vitro* assays have been made available (6–12). Nevertheless, failure to identify all active compounds and selection of numerous false-positive hits have recently been associated with the use of promastigotes in primary screenings, suggesting that host cell-mediated effects and stage-specific chemosensitivities should be addressed at early stages of drug discovery programs (13, 14). Testing on intramacrophage amastigotes, therefore, remains crucial, though challenging, given the lack of standardized methods that characterize the culturing of these parasite forms and the biological diversity exhibited by the different strains and species. Technically difficult and laborious, assessment of anti-leishmanial activity against the vertebrate host stage traditionally relies on microscopic quantification of parasite burdens (4, 5, 15, 16), with major problems related to data quality and poor performance. Establishment of parasite viability through staining procedures is difficult and error-prone, due to the uneven distribution of parasite loads throughout the sample and the difficulty to recognize individual amastigotes within heavily infected host cells. Recent progress in automated image-based screening assays (13, 17) and genetic engineering of *Leishmania* spp. (18–21) has opened important avenues for the high-throughput search of compounds targeting the intracellular stage. Nonetheless, advanced technology and intense manipulation are required to perform these assays, precluding their broad implementation and routine application to clinical isolates.

We report the development and validation of a simple, one-step assay for measuring drug activity against *Leishmania* intracellular amastigotes grown in human macrophages. Trypanothione reductase (TryR), an essential component of the kinetoplastid-unique thiol-redox metabolism (22, 23), was used to assess the viability of the *Leishmania* parasites by monitoring its 5,5′-dithiobis 2-nitrobenzoic acid (DTNB)-coupled reducing activity. This reaction combines the TryR-catalyzed reduction of trypanothione disulfide ($T[S]_2$) with its *in situ* regeneration through DTNB, re-

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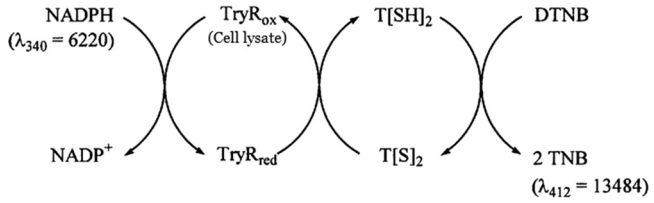

FIG 1 Schematic of the DTNB-coupled reaction. (Adapted from reference 24 with permission of the Biochemical Society.)

sulting in a colorimetric assay with improved sensitivity and efficiency (Fig. 1) (24). No cross-reactions with the host cells were observed, as a result of the poor affinity of substrate for the mammalian homologue. The assay was designed for microtiter plate format and colorimetric detection, enabling automation and high-throughput measurements with basic equipment. With its simplicity and robustness, this assay offers a valuable tool for the screening of new anti-leishmanial drugs, simplifying the method for assessing inhibitory activities at intracellular amastigote stage and improving accessibility to drug susceptibility testing with the ability to implement in most laboratories.

MATERIALS AND METHODS

Chemicals and drugs. All chemicals and drugs were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO), unless stated otherwise. Triton X-100 and $T[S]_2$ were obtained from BDH Laboratory Supplies (Poole, United Kingdom) and Bachem AG (Bubendorf, Switzerland), respectively. alamarBlue was purchased from AbD Serotec (Düsseldorf, Germany). The test compounds (VUF series of cathecolpyrazolinones and the dibenzylmethylene amine derivative IOTA 0058) are proprietary compounds of the VU University, Amsterdam, the Netherlands, and were provided within the framework of a collaborative project for development of new phosphodiesterase inhibitors (T4-302; TIPharma) (25). Amphotericin B and sodium stibogluconate were purchased from Merck KGaA (Darmstadt, Germany). Stock solutions of NADPH and $T[S]_2$ were prepared at a concentration of 8 and 10 mM, respectively (NADPH in Tris [0.5 M] buffer, pH 7.5; $T[S]_2$ in water), stored at −70°C, and freshly thawed before use. DTNB was dissolved in ethanol at a concentration of 25 mM prior to each experiment. Further dilutions of NADPH, $T[S]_2$, and DTNB were performed in Tris (0.05 M) buffer, pH 7.5. Stock solutions of the test drugs were prepared at concentrations of 5 to 10 mM in dimethyl sulfoxide (DMSO), water, or ethanol, according to the corresponding solubility.

Cell cultures. Promastigotes of *Leishmania donovani* (strains MHOM/SD/1968/1S and antimony-resistant MHOM/IN/2010/BHU814) and *Leishmania major* (strain MHOM/IR/1972/NADIM5) were maintained at 27°C in RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich Co.), 25 mM HEPES, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The human monocyte leukemia cell line THP-1 (ATCC no. TIB 202) was grown in RPMI 1640 medium supplemented with 10% FBS, 25 mM HEPES, 2 mM L-glutamine, and 1% antibiotics in a 5% CO_2 atmosphere at 37°C. Intramacrophage amastigotes of *L. donovani* were cultured as previously described (15), with minor modifications. Briefly, prior to infection, THP-1 monocytes (2.5×10^5 cells/ml) were differentiated into adherent, nondividing macrophage-like cells by a 48-h incubation with 10 ng/ml phorbol 12-myristate 13-acetate (PMA). After elimination of PMA by 3 washes, differentiated THP-1 cells were incubated overnight with stationary-growth-phase *L. donovani* promastigotes at a parasite/cell ratio of 10/1. Noninternalized promastigotes were then removed by washing adherent cells three times in excess medium, and the cultures were reincubated at 37°C in 5% CO_2 for an additional 72 h. All cell culture reagents were purchased from Gibco (Bleiswijk, the Netherlands), unless otherwise stated.

TryR-based assay. Assessment of TryR activity was performed in THP-1 cell and *Leishmania* parasite lysates. After a prewash with phosphate-buffered saline to remove potentially interfering compounds, samples were chemically lysed by 15 min incubation with a lysis buffer (200 μl/well), consisting of EDTA (1 mM), HEPES (40 mM), Tris (50 mM; pH 7.5), and Triton X-100 (2% vol/vol). Immediately prior to use, the buffer was supplemented with the protease inhibitor phenylmethanesulfonyl fluoride at a final concentration of 1 mM. TryR activity was measured in 75 μl of sample lysate, dispensed into the test well of a 96-well plate. NADPH (25 μl/well), $T[S]_2$ (75 μl/well), and DTNB (25 μl/well) were sequentially added to the sample lysate to yield final concentrations of 200, 75, and 100 μM, respectively. A blank was set for each sample, consisting of sample lysate supplemented with the reaction mixture described above, in which the substrate $T[S]_2$ had been replaced by Tris (0.05 M) buffer, pH 7.5. After a serial 27°C incubation ranging from few minutes to 3 h, absorbance was measured with an Infinite M200Pro multimode plate reader from Tecan (Männedorf, Switzerland) at a wavelength of 412 nm. The optical density, as measured in the blank, was subtracted from the corresponding sample signal, yielding the TryR activity responsible for 2-nitro-5-thiobenzoate (TNB^{2-}) production.

Promastigote drug susceptibility assay. Exponentially growing promastigotes, seeded in 96-well tissue culture plates at an initial concentration of 1×10^6/ml, were allowed to grow for 72 h at 27°C in the presence of a series of drug concentrations, ranging from 0 to a maximum of 10^{-3} M. Test drugs were serially diluted (in triplicate for each concentration) in promastigote culture medium supplemented with the corresponding drug solvent to yield equal concentrations of solvent over the dilution series. Untreated controls consisting of parasites incubated with medium only (with drug solvent) were included in triplicate in each test plate. Drug activity, as measured with the TryR-based assay, was compared with that obtained by the previously described alamarBlue method (7). For each test drug, plates for both assay methods were prepared in parallel with the same cells and medium. For the alamarBlue assay, 20 μl (10% vol/vol) of alamarBlue reagent was added to each well. After 5 h of incubation at 27°C, fluorescence was measured with a Tecan Infinite M200Pro multimode plate reader, with excitation and emission wavelengths of 560 and 590 nm, respectively, and a plate-tailored optimal gain setting. For the TryR-based assay, samples were processed as described above.

Intracellular amastigote drug susceptibility assay. THP-1 monocytes were plated in 16-well chamber slides (Lab-Tek, Nunc) or in 96-well culture plates for microscopic and enzymatic assessment, respectively. Differentiation and subsequent infection of cells were carried out as described above. After removal of noninternalized promastigotes by extensive washing with RPMI 1640 medium, parasitized cells were incubated with medium (untreated control) or a range of drug concentrations, as described for the promastigote drug assay. A negative control consisting of uninfected cells with no drug exposure was also included in each plate. After 72 h of incubation at 37°C in 5% CO_2, total parasite burdens (calculated as the percentage of infected macrophages × mean number of amastigotes per macrophage) were microscopically assessed on Field-stained slides and compared to the burdens of the untreated infected controls. At least 100 macrophages were counted per sample, and infection was judged to be adequate if 70 to 80% of the macrophages present in the untreated controls were infected. For the TryR-based assay, infection of cells and subsequent testing of drugs were carried out as described above. After 72 h of incubation at 37°C, 5% CO_2, cells were washed and lysed prior to being tested with the TryR-based assay. Results were expressed as the percentage of activity reduction in the treated sample versus the untreated control. No microscopic assessment of macrophage infection rate was performed on these samples.

Statistical analysis. The inhibition of parasite growth was determined by comparison of the signal produced by drug-treated parasites with that of untreated control parasites. Nonlinear regression analysis (GraphPad

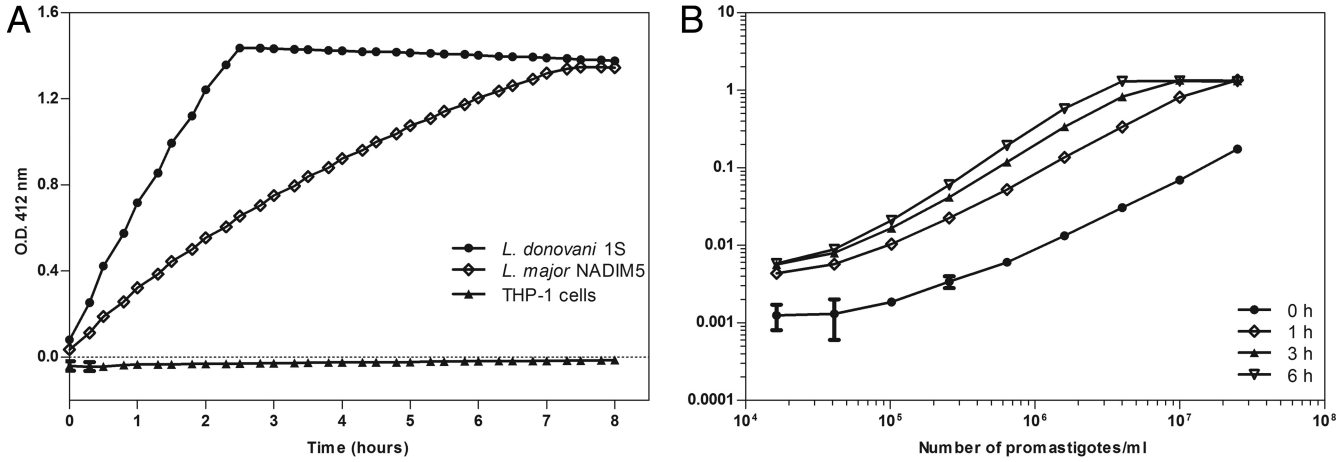

FIG 2 Time- and dose-dependent TryR activity. (A) Kinetics of TryR activity in parasite and cell lysates. Equal amounts (5×10^6/ml) of *Leishmania* promastigotes (*L. donovani* 1S and *L. major* NADIM5) and THP-1 cells were lysed, and their TryR-mediated reduction of DTNB was assessed at intervals of 15 minutes. (B) Dose-dependent TryR-mediated reduction of DTNB in *L. donovani* 1S promastigote lysate.

Prism, version 5.03; Software Inc., San Diego, CA) was used for curve fitting and calculation of 50% inhibitory concentrations (IC_{50}) and 90% inhibitory concentrations (IC_{90}). Analysis of correlation and agreement between data obtained from the TryR-based assay and microscopy was performed after transformation of assay results to log scale. Transformed data from the two assays were used for obtaining a scatter plot in which one set of assay results was plotted against the other on a log-log scale. The level of correlation between these data was assessed by the Pearson's correlation test (GraphPad Prism), in which the null hypothesis stated that no linear relationship linked the measurements obtained by the two methods (26). To determine the level of agreement between the two tests, the transformed data from both assays were graphed in a Bland-Altman plot (27) (GraphPad Prism), where the difference of IC_{50} values obtained for each compound by the two assays was plotted as a function of the mean IC_{50} between microscopy and the TryR-based assay. Two standard deviations (SD) of the mean IC_{50} difference between two assays were defined as the acceptable boundary range, as previously described (26), and were incorporated in the Bland-Altman plot to visualize the acceptable variation for the agreement. For validation of the TryR-based assay, the mean ratio (MR) and its confidence limits, the minimum significant ratio (MSR) and its limits of agreement (LsA), and the Z′ factor were calculated with Microsoft Excel software. The MR with its confidence limits and the MSR with its LsA were used to characterize the reproducibility in replicate experiments of average and individual compound potency estimates, respectively (28). The MR, whose ideal value should be 1.0, represents the average potency ratio across two concentration-response assay runs. When included within statistical limits of 1.0, the MR indicates no statistically significant differences in compound activity between runs. The MSR describes the largest potency ratio that can be considered a random change within a run of the assay, and its LsA identify statistical limits for individual potency ratios. Typically, an assay is considered acceptable if the MSR is small enough and both the LsA are close to 1.0. For our purpose, acceptance criteria were set at ≤ 3 for MSR and at 0.33 and 3.0 for the LsA, similar to what was previously described by Eastwood et al. (28). For assessment of Z′ factor values, the signal data yielded by untreated control *L. donovani* BHU814-infected and uninfected host cells were used. The Z′ factor, a characteristic parameter for the quality of the assay itself (29), is commonly used to validate the reliability of single-concentration HTS, as its values predict the rates of false positives and negatives to be expected with an assay. The categorization of screening assay quality by the value of the Z′ factor has been described elsewhere (29). Typically, a Z′ factor value >0.5 is defined as acceptable for an assay to be used in compound testing (26).

RESULTS

Development of a TryR-based assay to quantify *Leishmania* spp. A TryR-based assay for the quantification of *Leishmania* parasites was developed, adapting the previously described TryR DTNB-coupled reaction (24) for use with the nonpurified native enzyme contained in parasite lysates. The kinetics of the TryR-catalyzed reaction was monitored spectrophotometrically at 412 nm, after correcting for the non-trypanothione ($T[SH]_2$)-mediated reduction of DTNB. Free thiols stoichiometrically convert colorless DTNB into yellow TNB^{2-}, whose absorbance is commonly used to quantify the number of sulfhydrylic groups in biological samples. To monitor the $T[SH]_2$-mediated production of TNB^{2-} solely, a blank sample, consisting of lysate incubated with all reagents but substrate, was set for each sample, and its optical density was subtracted from the signal of the corresponding test sample. DTNB was reduced by the $T[SH]_2$ produced in lysates of *L. donovani* 1S and *L. major* NADIM5 promastigotes at a constant speed, until exhaustion of one or more reagents (Fig. 2A). Conversely, no reduction of $T[S]_2$ was observed with host cells (Fig. 2A), confirming the low affinity of the glutathione reductase for trypanothione-disulfide. Promastigotes of *L. donovani* and *L. major* exhibited strikingly different reaction rates in terms of DTNB reduction, highlighting species-specific features of the TryR metabolism. Under optimized conditions, a linear relationship ($R^2 = 0.9973$ to 0.9999) between plating density and absorbance was observed when different numbers of promastigotes (ranging from 4.1×10^4 to 2.5×10^7 parasites/ml, depending on the reaction time) were incubated with the reaction mixture for 0 to 6 h (Fig. 2B). Longer incubation times (3 to 6 h) resulted in increased sensitivity of the assay due to higher optical densities and, when linear, in enhanced signal differences between serial dilution points, due to increase of the curve slopes. However, they also caused the curves to plateau at higher parasite numbers, due to depletion of substrate and/or coenzyme. After 3 or more hours of incubation, only a small increase in absorbance was observed with 3×10^3 promastigotes, which therefore represents the lower detection limit of this assay.

Assessment of standard drug efficacies against *Leishmania* promastigotes. The suitability of the TryR-based assay for phar-

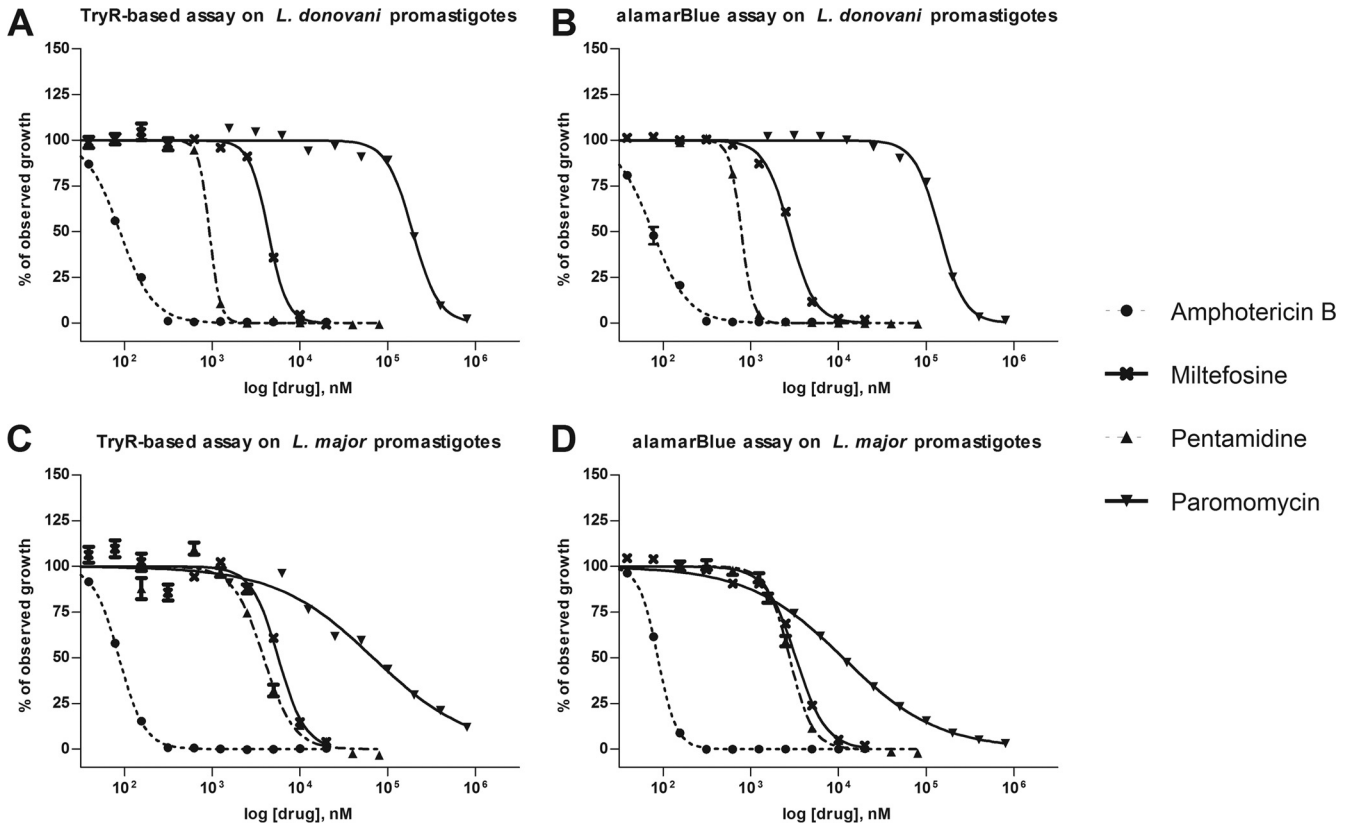

FIG 3 Dose-response curves of *L. donovani* (A and B) and *L. major* (C and D) promastigotes to four anti-leishmanial reference drugs (amphotericin B, miltefosine, pentamidine, and paromomycin), as measured by the TryR-based assay (A and C) and the conventional alamarBlue method (B and D). Values are normalized by use of the upper and the lower best-fit values as 100 and 0% responses, respectively, and are plotted as the means ± standard errors of the means for three replicates. Two independent determinations were performed with both methods run in parallel, and representative data of one of the two experiments are shown.

macological studies was initially confirmed on promastigotes, by assessing the efficacies of four anti-leishmanial reference drugs. Amphotericin B, miltefosine, pentamidine, and paromomycin were tested against promastigotes of *L. donovani* and *L. major*, two major species causing visceral and cutaneous leishmaniasis, respectively. The inhibitory activities of the four drugs were measured with the novel TryR-based assay and compared to the activities obtained with the conventional alamarBlue method. All drugs exhibited a dose-dependent effect on parasite proliferation, with dose-response curves and IC_{50} and IC_{90} values showing high correlation between the alamarBlue determination and the novel TryR-based assay (Fig. 3 and Table 1). Different susceptibilities to pentamidine isethionate were observed for *L. donovani* and *L. major* parasites, with the two methods being in agreement with each other.

Assessment of standard drug efficacies against *Leishmania* intracellular amastigotes. To validate the use of the TryR-based assay for assessing viability of the *Leishmania* intramacrophage forms, the activity of the four reference drugs was measured against *L. donovani* 1S and *L. major* NADIM5 intracellular amastigotes. Light microscopy was used as the conventional method to measure drug efficacies, due to incompatibility of the alamarBlue assay with the intracellular model. The TryR-catalyzed reaction was allowed to proceed for 3 h prior to measurement, because of

TABLE 1 Comparison of IC_{50} and IC_{90} values of four anti-leishmanial reference drugs against *L. donovani* and *L. major* promastigotes, as measured by the TryR-based assay and the alamarBlue method

Reference drug	IC_{50} (μM)[a]				IC_{90} (μM)[a]			
	L. donovani promastigotes		*L. major* promastigotes		*L. donovani* promastigotes		*L. major* promastigotes	
	TryR-based assay	alamarBlue method	TryR-based assay	alamarBlue method	TryR-based assay	alamarBlue method	TryR-based assay	alamarBlue method
Amphotericin B	0.133 ± 0.0410	0.135 ± 0.0475	0.143 ± 0.0647	0.140 ± 0.0671	0.227 ± 0.00735	0.195 ± 0.0443	1.03 ± 0.839	1.02 ± 0.844
Miltefosine	10.6 ± 6.16	11.8 ± 8.58	6.70 ± 1.07	4.24 ± 1.02	16.4 ± 9.10	23.6 ± 15.2	13.7 ± 0.420	8.52 ± 0.0585
Pentamidine	0.836 ± 0.159	1.64 ± 1.11	5.60 ± 1.63	6.51 ± 3.75	7.53 ± 1.44	5.12 ± 0.396	9.21 ± 0.135	18.2 ± 12.6
Paromomycin	161 ± 32.4	123 ± 18.7	123 ± 53.6	95.3 ± 84.7	292 ± 115	343 ± 46.2	581 ± 278	218 ± 33.0

[a] Values are shown with three significant digits and are the means ± standard errors of the means from two independent parallel determinations run in triplicate.

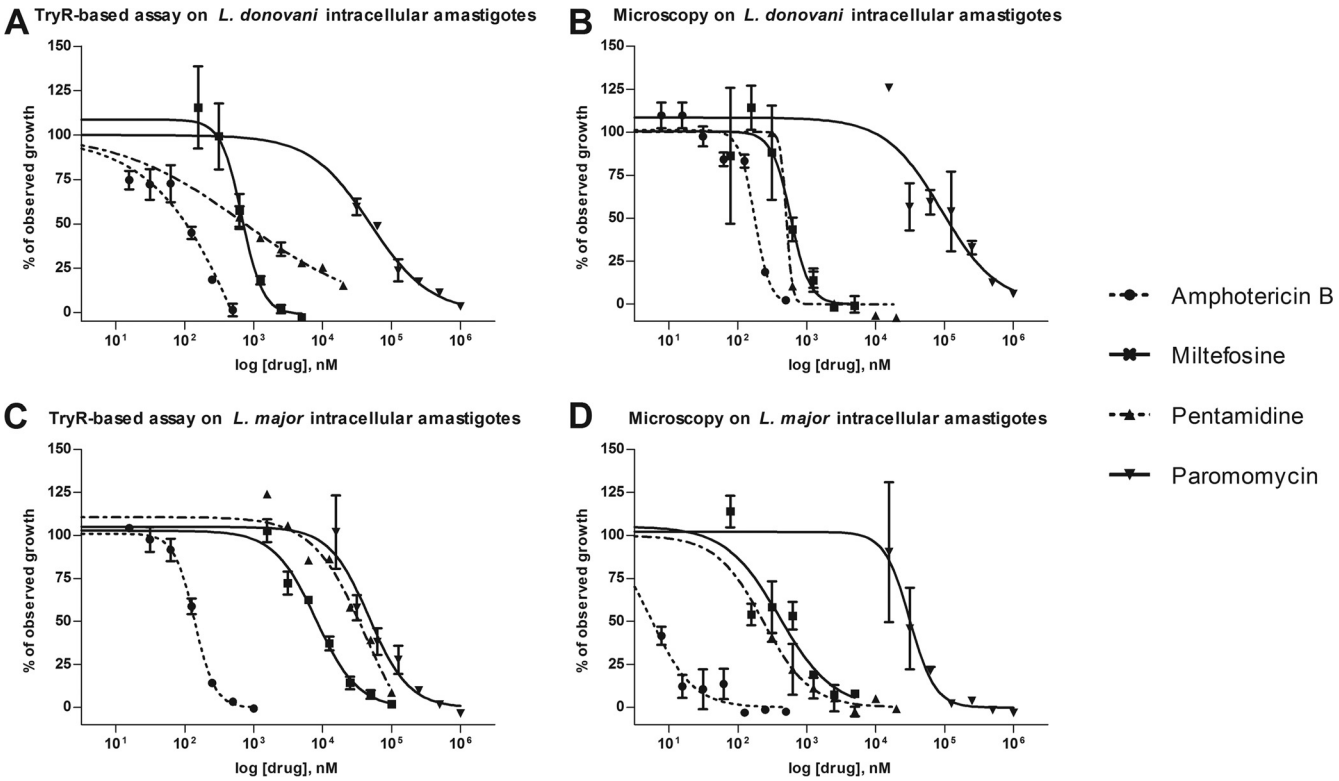

FIG 4 Dose-response curves of *L. donovani* (A and B) and *L. major* intracellular amastigotes (C and D) to four anti-leishmanial reference drugs (amphotericin B, miltefosine, pentamidine, and paromomycin), as measured by the TryR-based assay (A and C) and standard microscopy (B and D). Values are normalized by use of the upper and the lower best-fit values as 100 and 0% responses, respectively, and are plotted as the means ± standard errors of the means of three replicates for the TryR-based assay and two replicates for microscopy. Two independent determinations were performed with both methods run in parallel, and representative data from one of the two experiments are shown.

the lower parasite loads compared to the axenic tests. Similarly to the promastigote stage, the amastigote activity of the TryR reflected the dose-dependent effects of the drugs, resulting in growth curves and IC_{50} and IC_{90} values largely consistent with the microscopic assessments (Fig. 4 and Table 2). Species-specific chemosensitivities were observed with pentamidine isethionate, whose potency against *L. donovani* visceralizing forms was 20 times as high as against *L. major* ones (responsible for cutaneous leishmaniasis), similar to what was measured in promastigote stages.

Pharmacological and HTS validation. To assess the pharmacological relevance of this assay and analyze its robustness, two independent tests were performed with a 19-compound set and the corresponding activities blindly assessed by microscopy and the TryR-based assay (Table 3). The compound panel included a variety of chemicals with different potencies and mechanistic actions, ranging from TryR, protein kinase, and phosphodiesterase inhibitors (25) to G protein-coupled receptor ligands and various antiprotozoals, including a few anti-leishmanial reference drugs (Table 2). Overall, data assessed by microscopy resulted in noisier curves than data in the TryR-based assay, affecting the quality of fitting results and their reproducibility therein. As a consequence,

TABLE 2 Comparison of IC_{50} and IC_{90} values of four anti-leishmanial reference drugs against *L. donovani* and *L. major* intracellular amastigotes, as measured by the TryR-based assay and light microscopy

Reference drug	IC_{50} (μM)[a]				IC_{90} (μM)[a]			
	L. donovani intracellular amastigotes		*L. major* intracellular amastigotes		*L. donovani* intracellular amastigotes		*L. major* intracellular amastigotes	
	TryR-based assay	Microscopy	TryR-based assay	Microscopy	TryR-based assay	Microscopy	TryR-based assay	Microscopy
Amphotericin B	0.0606 ± 0.0280	0.100 ± 0.0780	0.414 ± 0.275	0.0100 ± 0.00150	0.363 ± 0.223	0.183 ± 0.124	0.763 ± 0.469	0.231 ± 0.177
Miltefosine	0.760 ± 0.355	0.708 ± 0.115	1.01 ± 0.173	0.930 ± 0.0310	3.37 ± 1.26	1.80 ± 0.609	3.25 ± 0.380	5.20 ± 0.576
Pentamidine	0.607 ± 0.0755	0.284 ± 0.237	5.91 ± 2.52	1.38 ± 0.344	5.46 ± 0.679	0.527 ± 0.102	204 ± 29.5	27.6 ± 10.5
Paromomycin	59.1 ± 9.97	77.0 ± 35.1	46.2 ± 3.09	32.6 ± 0.656	334 ± 93.7	507 ± 249	297 ± 91.2	189 ± 110

[a] Values are shown with three significant digits and are the means ± standard errors of the means from two independent parallel determinations run in triplicate for the TryR-based assay and in duplicate for microscopy.

TABLE 3 Comparison of IC_{50} and IC_{90} values of test drugs against *L. donovani* intracellular amastigotes, as measured by the TryR-based assay and light microscopy

Test drugs	IC_{50} (μM)[a]		IC_{90} (μM)[a]	
	TryR-based assay	Microscopy	TryR-based assay	Microscopy
Imipramine	15.6 ± 2.84	18.5 ± 0.640	29.7 ± 1.97	27.9 ± 3.30
Carmustine	15.2	8.80	66.0	41.2
H-89	7.30 ± 4.42	12.2 ± 2.33	75.2 ± 30.3	110 ± 21.0
Metergoline	8.18	17.1	22.0	41.4
Naloxonazine	5.67 ± 0.384	19.0 ± 5.97	27.9 ± 14.7	96.4 ± 21.3
VUF 11852	29.2 ± 1.02	20.2 ± 11.3	63.1 ± 0.240	54.0 ± 40.6
VUF 11854	25.6 ± 0.570	15.1 ± 1.59	59.9 ± 0.395	37.0 ± 12.7
VUF 11856	17.3 ± 3.17	30.8 ± 10.1	40.4 ± 4.64	66.7 ± 18.3
VUF 11857	15.0 ± 7.87	7.08 ± 0.870	104 ± 59.2	30.8 ± 19.8
VUF 13577	9.29 ± 0.732	7.20 ± 0.147	20.0 ± 0.380	12.1 ± 2.82
IOTA 0058	3.25 ± 0.0730	5.56 ± 1.26	18.7 ± 7.45	23.6 ± 14.2
Quinacrine	1.80 ± 0.365	0.522 ± 0.315	4.06 ± 1.74	4.15 ± 3.52
Eflornithine	13.0 ± 11.5	34.1 ± 14.6	127	176
Nifurtimox	23.9	23.9	38.3	37.9
Suramin	319	404	-	1344

[a] Values are shown with three significant digits and are the means ± standard errors of the means of two independent parallel determinations run in triplicate for the TryR-based assay and in duplicate for microscopy. For carmustine, metergoline, nifurtimox, and suramin, data obtained from one parallel determination run in triplicate for the TryR-based assay and in duplicate for microscopy are shown. For eflornithine, IC_{90} values could be derived from one parallel determination only, due to insufficient inhibition in one of the two tests. Insufficient inhibition for determining the IC_{90} value was observed for suramin, too, when it was tested with the TryR-based assay only.

estimation of IC_{50}s for compounds displaying weak anti-leishmanial activities and poorly fitted dose-response curves, such as eflornithine, metergoline, and carmustine, was not always feasible. Poor solubility of compound H-89 resulted in precipitation at the highest concentrations and very steep dose-response curves. Nifurtimox and suramin exhibited insufficient inhibition during the first test but yielded complete dose-response curves in the second run when higher concentrations were tested. The level of correlation and agreement between the TryR-based assay and the microscopic assessments was examined with the Pearson's correlation test and the Bland-Altman plot. The scatter plot (Fig. 5A) showed a significantly high level of correlation between the results from the two assays (coefficient correlation [r] = 0.88; $P < 0.0001$), with the line of best fit (slope = 0.97 ± 0.09) approximating the line of equivalence, which can be expected if the data from each assay were identical (i.e., the line $x = y$). In the Bland-Altman plot (Fig. 5B), all points were scattered around 0 and fell within the limits shown (upper limit of the agreement = 0.495; lower limit = −0.563), with a negative bias of −0.034 toward the TryR-based assay, indicating that data can be normalized from the TryR-based assay to microscopy by a factor of 0.95. No tendency toward a greater or smaller difference between the two methods was seen as the IC_{50}s increased.

For the TryR-based assay, the interassay activity variation for compounds ($n = 11$) devoid of solubility problems and exhibiting complete inhibitory curves over the tested dose range resulted in an MR of 1.10 with confidence limits of 0.88 and 1.39, indicating that there was no systematic difference in potency estimates between the two runs. Similarly, with an MSR of 2.70 and LsA of 0.55 and 2.98, and with no compound within the 11-compound subcohort used for statistical assessment lying outside the LsA, the assay met the acceptance criteria described above. To monitor for signal stability, the following measurements were performed. Three independent plates, each containing 30 replicates of uninfected and *L. donovani* BHU814-infected cells (edging wells were excluded), were sequentially measured over a 6-week period, enabling calculation of assay windows. Z′ factor values of 0.502, 0.559, and 0.646, respectively, were obtained for the three measured plates, confirming the good performance of the assay and its compliance with single-dose screen requirements (acceptance limit for the Z′ factor value was >0.5).

DISCUSSION

Critical to the success of drug discovery strategies against leishmaniasis is the use of the intracellular amastigote stage for primary screenings, which minimizes selection of falsely active compounds and prevents true hits from being undetected. New technologies for improving the quality and throughput of these assays have been developed but remain difficult to implement with clinical isolates or in nondedicated laboratories. Here, we describe a simple alternative assay for assessing the viability of

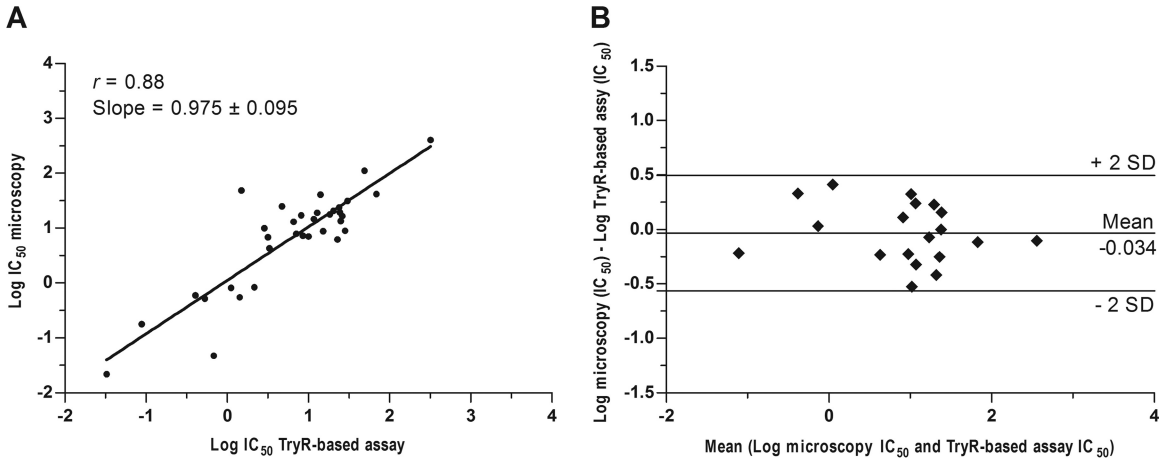

FIG 5 Analysis of agreement between the TryR-based assay and microscopy by a scatter plot (A) and a Bland-Altman plot (B) ($n = 34$ determinations). The mean difference of the Bland-Altman plot is −0.034. The upper and lower limits of agreement (mean ± 2 SD) are 0.495 and −0.563, respectively.

Leishmania intracellular forms and screening anti-leishmanial drugs at high throughput. The activity of a native, kinetoplastid-unique enzyme was measured by a one-step colorimetric assay and linearly related to the amount of parasites. TryR, the NADPH-dependent flavoprotein responsible for regeneration of the trypanothione pool, is essential to survival of trypanosomatids and, as such, is constitutively expressed throughout the parasite life cycle. Its absence from the mammalian host makes it a highly attractive target for drug development (23) and a valuable biomarker for parasite viability, as shown in this report. The previously described DTNB-coupled reaction for quantification of TryR activity in enzyme binding assays was applied to the complex environment of axenic and intramacrophage parasite cultures. The reaction was initiated after chemical disruption of cell membranes by a nonionic detergent-containing buffer, to ensure access of the substrate to the target enzyme ($T[S]_2$ is membrane impermeable). The low affinity of $T[S]_2$ for the glutathione reductase guarantees minimal cross-reactivity with the mammalian counterpart, which mainly contributes to the signal yield with its other thiols. Under optimized assay conditions, the $T[SH]_2$-mediated reduction of DTNB proved to be linear for up to 6 h, depending on the initial parasite inoculum (Fig. 2). Whereas for promastigote drug assays, the reaction was fast enough to enable immediate detection, more extended reaction times were required with intracellular amastigotes. The main reason behind this kinetic variability is most likely the >50-fold difference in parasite density between the two models, although stage-specific expression levels of TryR cannot be excluded. Differential rates of TryR-mediated reduction were also observed between *L. donovani* and *L. major* promastigotes, though with little effect on drug activities, suggesting that assay output may vary from one species to another, without affecting compound potency estimates. This does not exclude, however, the possibility that assay optimization might be required when the test is implemented with other *Leishmania* species or in other laboratory facilities.

A collection of structurally and pharmacologically diverse compounds was used to validate the assay, which performed robustly with drugs that are effective in the micro- and nanomolar ranges (Tables 1, 2, and 3). Reference anti-leishmanials exhibited highly correlated potencies when tested with the new TryR-based assay and a standard method (alamarBlue assay for promastigotes, microscopy for intracellular amastigotes) (Fig. 3 and 4), which corroborated literature data (7, 9, 13, 17, 20). Statistical analysis confirmed the high correlation and good agreement that characterized the results obtained with the TryR-based assay and microscopy, suggesting their interchangeability as drug screening methods. In agreement with previously published data (30–33), though not extensively addressed in the present study, was the observation that pentamidine and miltefosine exhibited differential effects toward different parasite species (*L. donovani* versus *L. major*) and developmental stages (promastigote versus amastigote), respectively. Comparable activities were also observed with the other tested compounds, including imipramine and carmustine, for which inhibitory activity toward TryR has been abundantly reported (34–36). It should be noted, however, that neither of the two drugs specifically targets the trypanosomatid enzyme, supporting the possibility that other mechanisms, as already reported in the literature (37), may have equally contributed to the mild toxicity displayed against the parasite (IC_{50}s = 15.6 μM and 12.6 μM, respectively) and its host cells. TryR-specific inhibitors with reduced toxicity for the mammalian cells should be tested to exclude possible interference with the assay, but at present, only a few compounds with the required features have been identified (36), and their access remains limited.

Compounds devoid of solubility problems and exhibiting complete dose-response curves at the tested concentrations yielded consistent IC_{50}s when assayed with the TryR-based test (but not with microscopy), as confirmed by meeting of the statistical parameters (MR, MSR, and LsA). Conversely, less reproducible potency estimates were obtained for drugs showing poor or excessive inhibition, possibly resulting from inaccuracies related to either poor IC_{50} curve fitting or initial parasite loads rather than from the assay itself. As for most cytotoxicity assays, determination of IC_{50}s by the TryR-based assay requires only an intraexperiment comparison of enzyme activities between the test samples and the controls, enabling the signals to be corrected for intertest variability. Nevertheless, differences in parasite growth can result in substantial effects on the IC_{50}s of some compounds, as previously described (38, 39). The cellular model used for validation of the TryR-based assay requires the *Leishmania* promastigotes to actively invade the macrophages and, upon conversion to amastigotes, proliferate therein. Though standardized in every step, the model is rather sensitive to variables like inoculum size, developmental stage, and handling procedures, each of which may influence the parasite growth rate. This was particularly true of *L. donovani* strain 1S, whose tendency to grow at 37°C intracellularly but also extracellularly resulted in variable and at times inadequate assay windows. However, when the recently isolated strain BHU814, for which much-improved infectivity rates were observed, was used, the Z′ values ranged from 0.50 to 0.65, indicating that robust performance of the assay to fulfill single-dose HTS requirements relies partly on the choice of appropriate *Leishmania* strains. Gene expression profiling of antimony-susceptible and -resistant strains recently highlighted important differences in the thiol metabolism of these isolates, with an overall, though at times inconsistent, overexpression of thiol-metabolizing enzymes among antimony-resistant parasites (40–43). While this might have contributed to the improved signal window yielded by the strain BHU814 (TryR overexpression, however, is yet to be demonstrated in this resistant strain), it is unlikely to interfere with drug activity assessment if the strains stably overexpress the TryR. Further evaluation with biochemically defined antimony-resistant and -sensitive isolates should be performed to exclude falsely enhanced IC_{50}s as a result of increased enzymatic activity.

Equally suitable for *Leishmania* promastigotes, albeit less convenient than existing methods, the TryR-based assay described here represents a significant advancement over the current techniques in assessing drug susceptibility at the intracellular amastigote stage, particularly in clinical isolates. Unlike standard microscopy, which remains the traditional screening method, the newly developed assay is fast, accurate, and operator independent. Timewise, the benefit is remarkable: while microscopic assessment of a duplicate 8-point drug dilution series requires a minimum of 1 h examination for a single compound, as many as 20 compound dilution series may be analyzed by the TryR-based assay in the same time frame (excluding the incubation step with the TryR reagents). The suitability of the assay for HTS applications, as confirmed by compliance with widely accepted statistical parameters rather than by actual use as high-throughput assay (prevented by the lengthy performance of the reference method),

makes it a valuable tool, along with other (semi)automated techniques (microscopy and reporter gene assays), compared to which it offers greater simplicity and applicability. Implementable in virtually every culture laboratory where a microplate reader is available, the test can be applied to both laboratory and field isolates, with no need for genetic manipulation, enabling drug susceptibility testing to be promptly and extensively performed. Its lowest detection limit makes it as sensitive as other *in vitro* screening methods (7, 9, 11, 19) but less powerful than some *Leishmania* recombinants, for which very low sensitivities (up to 10 promastigotes) have been described (20). Further limitations of the TryR-based assay include the relatively high costs associated with the use of the substrate $T[S]_2$ and the lack of morphological visualization, which characterizes the microscopic assessment. To this end, establishment of suitable, user-tailored signal thresholds for assessing minimum satisfactory infection rates in the control samples is a prerequisite for a future implementation of the assay as a microscopy replacement. Interestingly, recognition of the trypanothione pathway in other trypanosomatids, like *Trypanosoma cruzi*, whose life cycle also encompasses an intracellular stage, provide potential new applications to the assay for improving the search of new anti-trypanosomatid agents.

In conclusion, this report demonstrates the feasibility of a simple, rapid TryR-based assay for use in high-throughput drug screening against *Leishmania* intracellular amastigotes. Further optimization and implementation are required to determine the ultimate role of this method in anti-leishmanial drug discovery and establish its suitability for drug susceptibility testing.

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